Exhibit A

Cell, Vol. 95, 211–223, October 16, 1998, Copyright ©1998 by Cell Press

CREB1 Encodes a Nuclear Activator, a Repressor, and a Cytoplasmic Modulator that Form a Regulatory Unit Critical for Long-Term Facilitation

Dusan Bartsch,†‡ Andrea Casadio,† Kevin A. Karl,*† Paulo Serodio,† and Eric R. Kandel*†
*Howard Hughes Medical Institute
†Center for Neurobiology and Behavior
College of Physicians and Surgeons of Columbia University and
New York State Psychiatric Institute
New York, New York 10032

Summary

Although CREB seems to be important for memory formation, it is not known which of the isoforms of CREB, CREM, or ATF1 are expressed in the neurons that undergo long-term synaptic changes and what roles they have in memory formation. We have found a single *Aplysia CREB1* gene homologous to both mammalian CREB and CREM and have characterized in the sensory neurons that mediate gill-withdrawal reflex the expression and function of the three proteins that it encodes: CREB1a, CREB1b, and CREB1c. CREB1a is a transcriptional activator that is both necessary and, upon phosphorylation, sufficient for long-term facilitation. CREB1b is a repressor of long-term facilitation. Cytoplasmic CREB1c modulates both the short- and long-term facilitation. Thus, in the sensory neurons, *CREB1* encodes a critical regulatory unit converting short- to long-term synaptic changes.

Introduction

Both invertebrate and vertebrate nervous systems store information for short- and long-term memory by changing the strength of their synaptic connections (Bliss and Collingridge, 1993; for review, see Bailey et al., 1996). Studies in *Aplysia*, *Drosophila*, and mice suggest that short-term memory storage is accompanied by transient changes in the strength of synaptic connections by covalent modifications of preexisting proteins. By contrast, long-term memory storage is accompanied by enduring changes in synaptic strength that require both transcription and translation of genes (Montarolo et al., 1986; Nguyen et al., 1994). These persistent changes are, in some cases, accompanied by growth of new synaptic connections (Bailey and Chen, 1983).

In *Aplysia*, *Drosophila*, and rodents, the conversion of short- to long-term synaptic plasticity and memory formation requires an increase in intracellular cAMP and recruitment of the cAMP-dependent protein kinase A (PKA). In eukaryotic cells, transcriptional regulation in response to cAMP is primarily mediated by transcriptional activators and repressors of the CREB/ATF and CREM families. Consistent with a role for the CREB/ATF and CREM families of transcription factors in the regulation of genes responsive to cAMP, calcium, or neurotrophins, CRE sequences are often found in the upstream regulatory regions of genes transcriptionally responsive to these signaling pathways (for review, see Habener et al., 1995).

The initial molecular characterization of the role of cAMP, PKA, and CREB in synaptic plasticity came from studies of *Aplysia* where the synapse between the sensory and motor neurons of the gill-withdrawal reflex can be reconstituted in primary cell culture. In response to one pulse of serotonin (5-HT), a modulatory transmitter released by sensitization stimuli, this synapse undergoes cAMP-mediated short-term facilitation, whereas in response to five pulses of 5-HT this synapse undergoes long-term facilitation. The long-term facilitation requires transcription and translation and is selectively blocked by injection of CRE oligonucleotides into the nucleus of the sensory neurons (Dash et al., 1990). Furthermore, repeated pulses of 5-HT will activate in these sensory neurons a CREB reporter gene (Kaang et al., 1993). The central role of CREB in long-term memory was further demonstrated in studies of olfactory memory in *Drosophila*. In transgenic flies, the induced expression of a dCREB2b repressor selectively blocked translation-dependent long-term memory (Yin et al., 1994). Conversely, overexpression of the dCREB2a activator facilitated memory so that a task that normally requires multiple spaced training sessions was acquired in one training trial (Yin et al., 1995a). Finally, mice with disrupted expression of α and δ isoforms of CREB (Hummler et al., 1994) also show impairment in both LTP and long-term memory (Bourtchuladze et al., 1994).

Despite evidence that CRE-binding proteins are important components of a general switch that converts short-term to long-term synaptic and behavioral plasticity, it is not known which of the many CREB/ATF or CREM protein isoforms are involved in the specific cells that store particular forms of long-term memory and what role each of these isoforms has in the storage process. This lack of information not only reflects the difficulty in localizing and studying the critical cells that participate in memory storage, but also the complexity of CREB/ATF and CREM genes in higher eukaryotes (for review, see Habener et al., 1995). In both mammals and *Drosophila*, *CREB* mRNAs undergo extensive splicing (Waeber et al., 1991; Ruppert et al., 1992; Yin et al., 1995b). In addition to the multiple isoforms of CREB, there is at least an equal number of CREM isoforms (Foulkes et al., 1991; Molina et al., 1993). Given the variety of CREB/ATF and CREM isoforms and the possibility of functional compensation between them, it becomes particularly important to know which specific isoforms are expressed and to determine what role, if any, each of these isoforms plays in the specific cells that participate in memory storage.

To examine the role of CREB protein isoforms in synaptic plasticity in mature *Aplysia* sensory neurons, we cloned an *Aplysia CREB1* gene that appears to be the only member of the CREB, CREM, and ATF1 family of genes expressed in *Aplysia* neurons. We find that three

‡To whom correspondence should be addressed (e-mail: db579@columbia.edu).

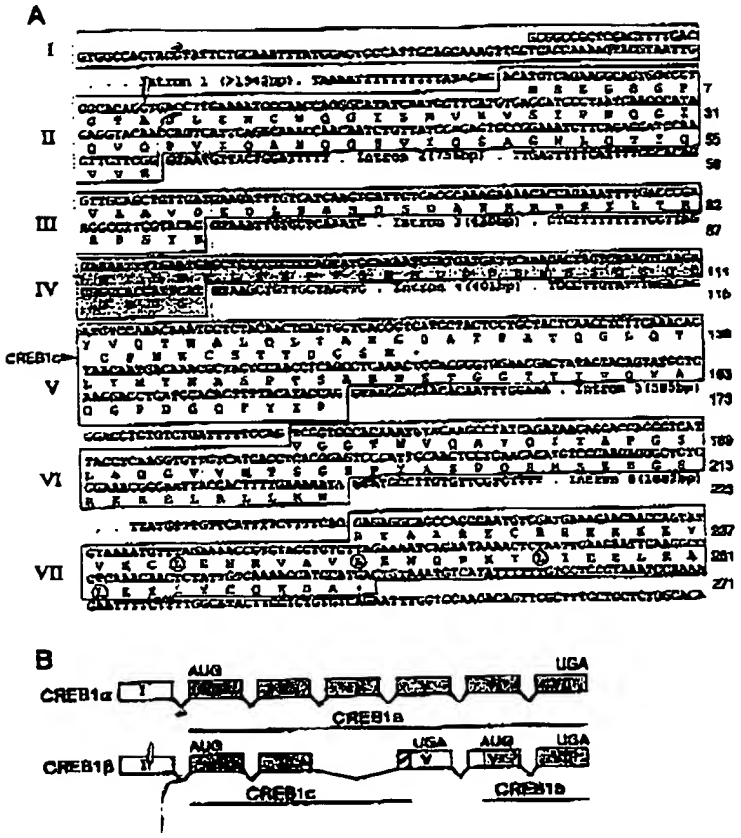

Figure 1. Nucleotide Sequence of the *Aplysia CREB1* Gene and Amino Acid Sequences of the *Aplysia* CREB1 Protein Isoforms

(A) CREB1 protein sequences were deduced from DNA sequences of *Aplysia* genomic clones and *CREB1α* and *CREB1β* cDNAs from a sensory neuron cDNA library. Exons are indicated by the roman numerals on the left side. The partial nucleotide sequence annotated as exon I was derived from the longest 5′ end of *CREB1α* cDNA. The intron boundaries (GT and AG) are bold. Only partial intron sequences and partial untranslated 3′ end of cDNAs are shown. In both *CREB1α* and *CREB1β* cDNAs, the first ATG is surrounded by Kozak sequence (Kozak, 1986b) and was assigned as the translation start. The numbering of the protein sequence on the right side starts with this first methionine as #1 and refers to the CREB1a protein isoform. *CREB1β* mRNA is generated by splicing-out of exon IV (shaded) and encodes the CREB1c and CREB1b protein isoforms. The C-terminal end of CREB1c is indicated by the arrowhead. The ATG for methionine 198 (referring to the CREB1α protein, bold) is also surrounded by the Kozak sequence and is the putative initiation codon for the CREB1b protein isoform translated from the *CREB1β* mRNA. The leucine residues forming the leucine zipper are circled.

(B) Schematic organization of the *Aplysia CREB1α* and *CREB1β* mRNAs. Exons, numbered I–VII, are shown as boxes, introns as connecting lines. The coding regions are shaded; noncoding regions are white. The new coding sequence in CREB1c generated by alternative splicing of *CREB1β* is striped. The open reading frame for individual CREB1c protein isoforms are underlined.

isoforms of CREB1 play a distinct role in synaptic plasticity in the sensory neurons. CREB1a protein is an activator that is both necessary and sufficient for long-term facilitation. CREB1b is a repressor of CREB1a and long-term facilitation. CREB1c is a cytoplasmic polypeptide that modulates both short-term and CREB1a-mediated long-term facilitation.

Results

CREB1 Gene Encodes Three Proteins Homologous to Mammalian CREB and CREM

We cloned two mRNAs expressed in *Aplysia* sensory neurons: *CREB1α* and *CREB1β*. The genomic sequence of *Aplysia CREB1* indicates that these two transcripts are generated by alternative splicing and that the 88 nucleotide insertion in *CREB1α* corresponds to the spliced-in exon IV (Figure 1). The longest open reading frame (ORF) in the *CREB1α* cDNA encodes a CREB1a polypeptide of 271 amino acids, which has sequence homology to both mammalian CREB and CREM proteins (Foulkes et al., 1991; Ruppert et al., 1992). The first ORF in the *CREB1β* mRNA encodes the 99–amino acid CREB1c polypeptide in which the C-terminal 12 amino acids are different from CREB1a (Figure 1).

CREB1a is 95% homologous to mammalian CREB and CREM proteins in its C-terminal DNA binding and dimerization domain (bZIP) and its phosphorylation domain (P box). The key regulatory phosphorylation consensus sites in the P box (Gonzalez et al., 1989) are conserved between *Aplysia* CREB1a and mammalian CREBs. The S85 (corresponding to S133 in mammalian CREB) and the surrounding recognition sequences for PKA, CaMK, and RSK2 are also conserved. Interestingly, similar to *Drosophila* dCREB2a (Yin et al., 1995a), the TB1 preserves the GSK3 phosphorylation site, homologous to S129 in mammalian CREBs (Fiol et al., 1994). In contrast to *Drosophila* dCREB2a and *Hydra* CREB (Galliot et al., 1995), the P box in *Aplysia* CREB1a contains the CAMKII phosphorylation site S94, homologous to S142 in mammalian CREB (Sun et al., 1994; Figure 1A).

In addition to being homologous to mammalian CREB proteins, *Aplysia* CREB1a is also homologous to mammalian CREM proteins in its P box (E92), bZIP domain (L218), and other motifs (for example, amino acids 56–68 and 204–206, Figure 1). However, *Aplysia CREB1* gene does not have a second bZIP domain, a feature typical for CREM genes. The *Aplysia CREB1* gene most likely resembles an evolutionarily early form of the gene that duplicated in higher eukaryotes to form the CREB and CREM genes. Consistent with this idea, *CREB1* appears to be the only CREB/CREM/ATF-1–like gene in the *Aplysia* genome. We failed to identify any additional

genomic sequences with significant homology to *CREB1*, *CREM*, or *ATF1* using PCR analysis, screening of genomic library, and Southern blot hybridization with the *CREB1a* cDNA probe at low stringency.

The *CREB1α* mRNA Encodes the CREB1a Activator

The *CREB1α* mRNA encodes a CREB1a protein with a predicted molecular weight of 29 kDa, which migrates at 40 kDa in SDS gels (Figures 3B and 3C). *Aplysia* CREB1a forms homodimers in the yeast two-hybrid system, and Gal4-CREB1a fusion protein activates transcription in yeast (data not shown). In addition, bacterially expressed recombinant CREB1a protein forms homodimers and binds to CRE in vitro (Figure 2B).

Transient transfection assays in F9 cells show that *CREB1α* mRNA encodes a PKA-dependent transcriptional activator (Figure 2A). Similar to mammalian CREB proteins, the mutation of S85 to A85 (S85A mutant, homologous to S133A in mammalian CREBs) generates a dominant-negative CREB1a mutant that inhibits CREB1a-mediated transactivation in transfection assays. Consistent with the conservation of the P box in CREB1a, recombinant CREB1a protein is a substrate for in vitro phosphorylation on S85 by PKA, CaMKII, and PKC (data not shown). Thus, the *Aplysia CREB1* gene encodes a protein, CREB1a, that is structurally and functionally homologous to the mammalian CREB transactivators.

The *CREB1β* mRNA Encodes the CREB1b Repressor and CREB1c Polypeptide

The removal of exon IV in the *CREB1β* mRNA by alternative splicing generates a frame shift, creating a new open reading frame that adds 12 new amino acids after R87 of the putative CREB1c polypeptide and is followed by a termination codon in exon V (Figure 1).

CREB1c does not contain a bZIP domain with the nuclear localization signal and is unable to bind DNA or form dimers (data not shown). In transient transfection assays in F9 cells, coexpression of the CREB1c ORF (nucleotides 1–300) does not affect CREB1a-mediated CRE transactivation. In transfected F9 cells, CREB1c is localized predominantly perinuclearly in the cytoplasm (Figure 2C). *Aplysia* CREB1c is a substrate for phosphorylation in vitro by PKA and PKC on S85, but although the CaMKII phosphorylation site surrounding S85 in CREB1c is intact, CREB1c is not phosphorylated by CaMKII in vitro (data not shown). The frame shift generating CREB1c adds 12 new amino acids after R87 and removes the S94 and S95 of CREB1a P box. Interestingly, in mammalian CREBs, the S94 homolog S142 is a substrate for CaMKII phosphorylation, which inhibits CREB activity (Sun et al., 1994).

CREB1b is translated from the second ORF in *CREB1β* mRNA (Figure 1B) and is most likely generated by internal initiation of translation, putatively at M196 (referring to CREB1a protein sequence, Figure 1A). The CREB1b product of in vitro translation of full-length *CREB1β* mRNA comigrates in gel with the product of truncated *CREB1β* (502–733) mRNA. CREB1b contains the C-terminal bZIP DNA binding and dimerization domain of CREB1a but does not contain its P box or activation domains and is not phosphorylated by PKA, CaMKII, or PKC in vitro (data not shown).

In contrast to CREB1c, CREB1b forms homodimers as well as heterodimers with CREB1a, both of which bind to CRE in vitro (Figure 2B). Transient transfections in F9 cells indicate that *CREB1β* mRNA encodes a repressor of CREB1a-mediated transactivation. Cotransfection of CREB1b ORF (nucleotides 502–733) and *CREB1α* cDNA indicates that CREB1b represses CREB1a-mediated transactivation of the CRE reporter (Figure 2A).

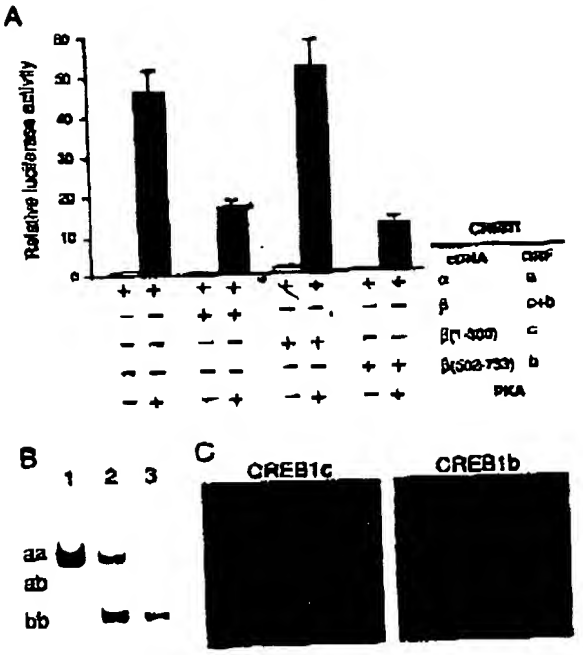

Figure 2. The *CREB1α* mRNA Encodes the Transcriptional Activator CREB1a and the *CREB1β* mRNA Encodes the CREB1b Repressor and Cytoplasmic CREB1c Polypeptide

(A) The *CREB1α* cDNA encodes a PKA-dependent CREB1a transcriptional activator; the *CREB1β* mRNA encodes the CREB1b repressor of CREB1a. F9 cells were transiently transfected with 0.5 μg 5xCRE-luc reporter plasmid, with or without PKA, along with 1 μg of each of the indicated RcRSV plasmids expressing either the full-length *CREB1α* cDNA, full-length *CREB1β* cDNA, or deletion mutants of *CREB1β* cDNA with the CREB1c ORF (*CREB1β* 1–300) and CREB1b ORF (*CREB1β* 502–733). Luciferase expression was normalized to β-galactosidase activity of cotransfected 0.2 μg RSV-lacZ plasmid. Relative luciferase activity was calculated by comparing the activities measured in cotransfection experiments to the activity of RcRSV-*CREB1α* alone (arbitrarily set as one). Each bar represents the mean of at least 12 independent transfections ± SEM.

(B) CREB1a and CREB1b bind CRE as homo- and heterodimers. Purified recombinant, bacterially expressed CREB1a (1), CREB1b (3), and their 1:1 mixture (2) were incubated with ^{32}P-labeled CRE and separated in native PAGE (EMSA). The positions of CREB1 homo (aa, bb) and heterodimers (ab) are indicated. CREB1c does not bind CRE (not shown).

(C) Subcellular localization of 6His-tagged CREB1c and CREB1b proteins in transiently transfected F9 cells 72 hr after transfection. The localization of the proteins was visualized by immunocytochemistry with anti-6His antibody.

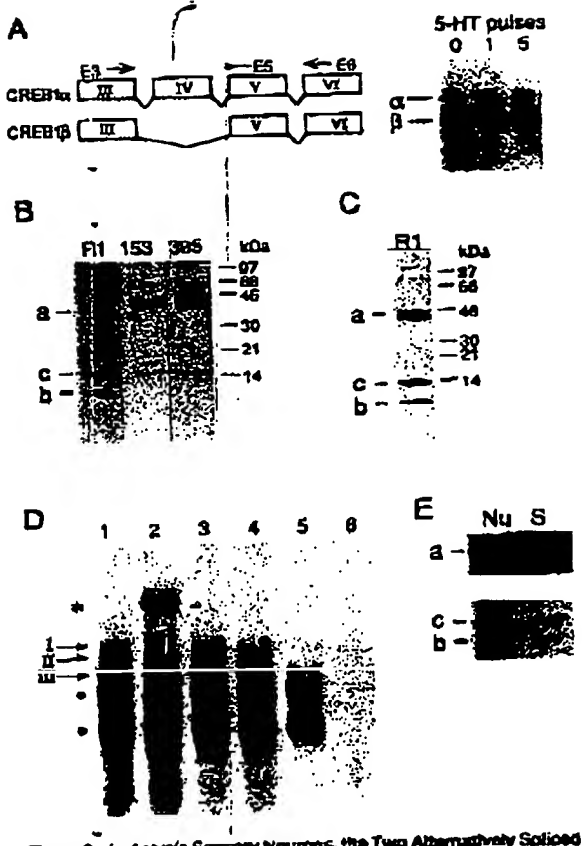

Figure 3. In *Aplysia* Sensory Neurons, the Two Alternatively Spliced CREB1 mRNAs, *CREB1α* and *CREB1β*, Encode Three Proteins: CREB1a; CREB1b, and CREB1c

(A) Schematic structures of *CREB1α* and *CREB1β* mRNAs expressed in *Aplysia* sensory neurons. The exons are labeled with roman numerals, and the positions of exon III-specific (E3), exon VI-specific (E6) primers (used for RT-PCR) and the exon V-specific (E5) primer are indicated. *Aplysia CREB1α* and *CREB1β* mRNAs were amplified by RT-PCR using the primers E3 and E6 from cultures of approximately 200 sensory neurons exposed to zero, one, or five pulses of 5-HT (10 μM). *CREB1α* (α, 224 bp) and *CREB1β* (β, 158 bp) PCR products were separated on 3% agarose gel and visualized by hybridization with ^{32}P-labeled (*) E5 oligonucleotide. *CREB1β* mRNA is more abundant than *CREB1α* mRNA in *Aplysia* sensory neurons, and the ratio of *CREB1α*/*CREB1β* mRNA does not change with 5-HT exposure.

(B) CREB1a, CREB1b, and CREB1c proteins are expressed in *Aplysia* neurons. Proteins isolated from *Aplysia* CNS (20 μg) were separated by SDS-PAGE, electroblotted, and probed with affinity-purified anti-CREB1 antibody (R1), anti-P box CREB1 (153), and anti-phospho-P-box CREB1 (395) antibodies. The positions of CREB1 isoforms (a, b, c) are indicated.

(C) CREB1a, CREB1b, and CREB1c proteins are expressed in *Aplysia* sensory neurons. Proteins isolated from six pleural sensory neuron clusters separated by SDS-PAGE were electroblotted and probed with the affinity-purified CREB1 (R1) antibodies. The positions of CREB1 isoforms are indicated.

(D) Three CRE binding complexes containing CREB1 proteins can be detected in *Aplysia* sensory neurons. Nuclear extract from *Aplysia* sensory neurons were incubated with ^{32}P-labeled (1) CRE, (2) CRE + anti-CREB1 antibody R1, (3) CRE + anti-ApC/EBP antibody, (4) CRE + 50× molar excess of mutated CRE competitor, (5) CRE + 5× molar excess of specific CRE competitor, (6) CRE + 50× molar excess of specific CRE competitor, and the DNA/protein complexes detected by EMSA. The arrows indicate the positions of three CRE binding complexes specifically supershifted by anti-CREB1 antibody (*) in lane 2.

(E) In *Aplysia* neurons, CREB1a and CREB1b proteins are nuclear and the CREB1c protein is cytoplasmic. Cytoplasmic (C) and nuclear (Nu) fractions from *Aplysia* neurons were separated by SDS-PAGE, electroblotted, and probed with the affinity-purified CREB1 (R1) antibodies. The positions of CREB1 isoforms are indicated.

These results indicate that two mRNAs transcribed from the *CREB1* gene encode three proteins. The *CREB1α* mRNA encodes the CREB1a transcriptional activator, and the bicistronic *CREB1β* mRNA encodes two additional CREB1 isoforms: the first ORF encodes CREB1c that lacks the bZIP domain and has a P box with modified kinase affinity; the second ORF encodes CREB1b, a transcriptional repressor.

In *Aplysia* Neurons, CREB1a and CREB1b Are Nuclear Proteins and CREB1c Is Cytoplasmic

We next analyzed whether both *CREB1α* and *CREB1β* mRNAs are expressed in sensory neurons. Using RT-PCR with primers derived from *Aplysia* CREB1 exon III and exon VI sequences, we found that sensory neurons express both mRNAs. In sensory neurons, the *CREB1β* mRNA is approximately ten times more abundant than *CREB1α*, and the *CREB1α*/*CREB1β* mRNA ratio does not change with one or five pulses of 5-HT (Figure 3A).

Western blotting of extracts from *Aplysia* sensory neurons with affinity-purified anti-CREB1 antibodies (R1) revealed three polypeptides with apparent molecular weights corresponding to CREB1a, CREB1b, and CREB1c (Figure 3C). Affinity-purified antibodies against the P box peptide of CREB1a (153) as well as anti-phospho-P box peptide antibodies (395) recognize CREB1a and CREB1c proteins (Figure 3B). In nuclear extracts from *Aplysia* sensory neurons, the gel-shift assay detects three complexes binding to the CRE oligonucleotide that are supershifted by the anti-CREB1a (R1) antibody (Figure 3D). These data indicate that the *Aplysia* sensory neurons express all three CREB1 isoforms: CREB1a, CREB1b, and CREB1c.

We next analyzed the subcellular localization of CREB1 protein isoforms in *Aplysia* neurons. In particular, we were interested in the localization of CREB1c, which lacks a nuclear localization signal and is found predominantly perinuclearly in the cytoplasm of transfected F9 cells (Figure 2C). Western blots of nuclear and cytoplasmic fractions of *Aplysia* neurons probed with anti-CREB1 antibodies (R1) indicate that CREB1c protein is cytoplasmic, whereas CREB1b and CREB1a are nuclear proteins (Figure 3E).

CREB1a Is Necessary for Long-Term Facilitation

To investigate the role of CREB1 in long-term facilitation, we first injected polyclonal anti-CREB1 antibody into the sensory neurons prior to exposing the cultures to five pulses of 5-HT. Whereas five pulses normally induce long-term facilitation, injection of anti-CREB1 antibody 1 hr before the 5-HT exposure completely blocked long-term facilitation (Figure 4B). Injection of the same antibody had no effect on short-term facilitation or basal synaptic transmission (Figure 4A).

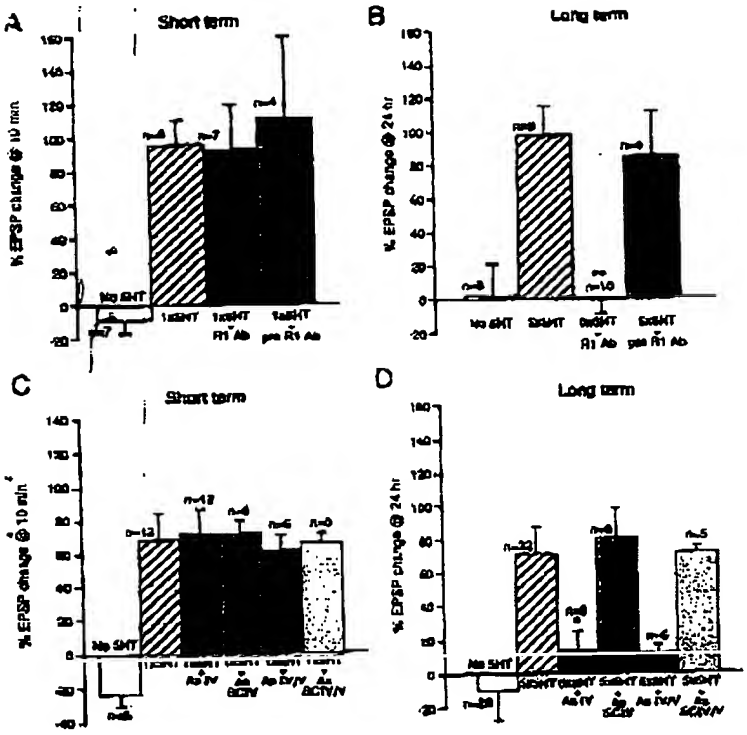

Figure 4. CREB1 Expression Is Necessary for Long-Term Facilitation

(A and B) Injection of anti-CREB1 antibodies into sensory neurons does not affect short-term facilitation but blocks long-term facilitation in sensory-motor synapses. Bar graphs represent the effect of anti-CREB1 antiserum (R1 Ab) and preimmune serum (pre R1 Ab) injection on short-term (A) or long-term facilitation (B). The height of each bar corresponds to the mean percentage change ± SEM in EPSP amplitude tested 10 min after one pulse of 5-HT (A) or 24 hr after five pulses of 5-HT (B). **, $p < 0.01$ compared with noninjected neurons exposed to five pulses of 5-HT.

(C and D) Injection of As IV and As IV/V antisense oligonucleotides targeting *CREB1α* mRNA does not affect short-term facilitation but blocks long-term facilitation. Bar graph represents the effect of injection of DNA oligonucleotides on short-term (D) and long-term (E) facilitation. The height of each bar corresponds to the mean percentage change ± SEM in EPSP amplitude tested 10 min after one pulse of 5-HT (D) and 24 hr after five pulses of 5-HT (E). *, $p < 0.05$ compared with noninjected neurons exposed to five pulses of 5-HT.

We next injected, into the sensory neurons, an antisense oligonucleotide (As I/II) complementary to the sequence surrounding the first putative initiation codon. The As I/II inhibited long-term facilitation when injected 4 hr prior to five pulses of 5-HT (21.99% ± 13.42%, n = 11). By contrast, cells injected with a scrambled oligonucleotide (As SCI/II) paired with five pulses of 5-HT underwent the same increase in synaptic strength (99.84% ± 14.53%, n = 6) evident in noninjected cells (103.84% ± 19.19%, n = 10). Short-term facilitation, induced by a single pulse of 5-HT, was not effected by injection of As I/II or As SCI/II and was comparable to short-term facilitation in uninjected control neurons. Oligonucleotide injection did not affect basal synaptic transmission (data not shown).

To investigate further the specific role of CREB1a in long-term facilitation, we injected an antisense oligonucleotide (As IV) that specifically targets sequences corresponding to exon IV in *CREB1α* mRNA and therefore interferes selectively with CREB1a expression. Injection of As IV inhibited long-term facilitation when injected 2, 4, or 6 hr before five pulses of 5-HT (35.51% ± 17.72%, n = 12; 13.45% ± 11.88%, n = 8; 17.70% ± 8.53%, n = 12). In contrast, cells injected with scrambled oligonucleotide (As SCIV) 4 hr before five pulses of 5-HT showed an increase in synaptic strength comparable to that of noninjected cells (Figure 4D). Short-term facilitation was comparable in As IV–injected, As SCIV–injected, and uninjected neurons (Figure 4C).

Finally, we injected an antisense oligonucleotide (As IV/V) that specifically targets *CREB1α* mRNA and interferes with the expression of CREB1a by binding to the boundary between exons IV and V. Injection of As IV/V again selectively blocked long-term facilitation (Figure 4D). In contrast, injection of neither As IV/V nor the scrambled As SCIV/V oligonucleotide affected short-term facilitation (Figure 4C).

These experiments indicate that CREB1 proteins, and specifically CREB1a, are necessary for the induction of long-term facilitation.

CREB1a Is Limiting for Long-Term Facilitation

To investigate further the role of CREB1a in the induction of long-term facilitation, we purified recombinant wild-type CREB1a and mutant S85A CREB1a proteins from *E. coli* and exposed them to PKA in vitro prior to injecting them into sensory neurons. We then examined the effect of injecting phosphorylated or unphosphorylated CREB1a on basal synaptic transmission, short-term, and long-term facilitation (Figure 5).

Injection of recombinant CREB1a, either phosphorylated or unphosphorylated, had no effect on basal synaptic transmission, short-term facilitation, or long-term facilitation. These results indicate that long-term facilitation produced by five pulses of 5-HT is saturated at 24 hr. To determine whether CREB1a could rescue the inhibition of long-term facilitation caused by injection of antisense oligonucleotide targeting the *CREB1α* mRNA, we coinjected the As IV/V oligonucleotide together with CREB1a protein and applied five pulses of 5-HT. CREB1a injection rescues the long-term facilitation inhibited by antisense targeting of CREB1a and indicates that this block was caused by depletion of CREB1a in *Aplysia* neurons. In contrast, long-term facilitation induced by

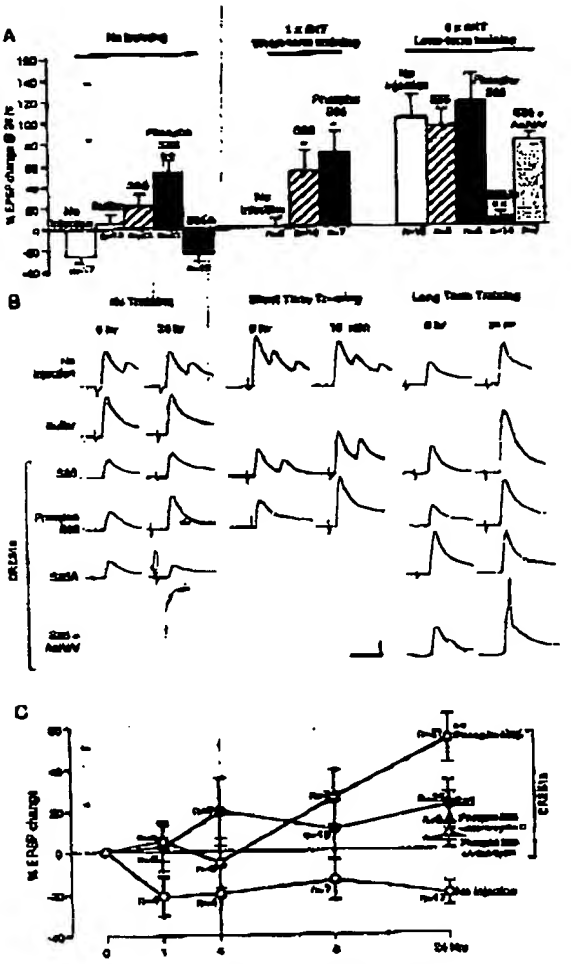

Figure 5 Phosphorylated CREB1a Is Sufficient for the Induction of Long-Term Facilitation

(A) Bar graph represents the effects of the injection of recombinant CREB1a protein phosphorylated by PKA (Phospho S85), nonphosphorylated (S85), or mutated in the PKA phosphorylation site (S85A) into sensory neurons. Cultures were exposed to five pulses of 5-HT (long-term training), one pulse of 5-HT (short-term training), or not exposed to 5-HT (no training). Injection of phosphorylated CREB1a induced long-term facilitation in the absence of any 5-HT treatment (**, $p < 0.01$; *, $p < 0.05$, compared with cells injected with the buffer solution). Injection of either phosphorylated or nonphosphorylated CREB1a did not affect long-term facilitation induced by five pulses of 5-HT, but coinjection of phosphorylated CREB1a with *CREB1a*-targeting antisense/RNA IVN reduced the block of long-term facilitation induced by five pulses of 5-HT. In contrast, injection of mutant CREB1a (S85A) blocked long-term facilitation induced by five pulses of 5-HT (**, $p < 0.01$ as compared to noninjected cells).

(B) Examples of EPSPs recorded at indicated time points in neurons injected with CREB1a. Calibration bars are 10 mV; 60 ms.

(C) CREB1a protein phosphorylated at S85 induces long-term facilitation in the absence of short-term facilitation. Time course of facilitation induced by CREB1a injection into sensory neurons in the absence of 5-HT treatment. Injection of CREB1a did not induce short-term facilitation 10 min after injection (not shown). Facilitation induced by the injection of phosphorylated CREB1a was detected after 4–6 hr and increased with time (**, $p < 0.01$ as compared with noninjected cells and cells injected with unphosphorylated CREB1a). This long-term facilitation was abolished by inhibitors of both mRNA (actinomycin D) and protein (anisomycin) synthesis.

five pulses of 5-HT was selectively blocked by injection of a dominant-negative mutant S85A CREB1a.

To determine whether CREB1a is limiting for the conversion of short- to long-term facilitation, we next examined the effect of injecting CREB1a paired with only a single pulse of 5-HT, which normally produces short-term facilitation only lasting minutes. When injected 1 hr before one pulse of 5-HT, both the unphosphorylated and phosphorylated CREB1a induced long-term facilitation that was at least 50% of that normally expressed after five pulses of 5-HT, thus indicating that the CREB1a is limiting in the initiation of the long-term process (Figure 5A).

Phosphorylated CREB1a Is Sufficient for Long-Term Facilitation

To ask whether CREB1a is sufficient to induce long-term facilitation by itself, without exposing the neurons to 5-HT, we injected PKA-phosphorylated CREB1a and found that it induced significant long-term facilitation even without 5-HT exposure (Figure 5A). This induction of long-term facilitation by phosphorylated CREB1a was abolished by application of either the RNA synthesis inhibitor actinomycin D or the protein synthesis inhibitor anisomycin (Figure 5C). To determine whether phosphorylation of S85 was required for the induction of long-term facilitation, we injected unphosphorylated CREB1a and found that it induced about 30% of long-term facilitation induced by phosphorylated CREB1a. Since the injection of the dominant-negative mutant S85A CREB1a produced a depression at 24 hr, similar to that seen in uninjected cells, we think that the facilitation produced by the injection of unphosphorylated CREB1a is likely the result of subsequent intracellular phosphorylation of CREB1a by second messenger pathways induced by the microelectrode penetration and Ca^{2+} influx.

Figure 5C shows the time course of the synaptic changes following CREB1a injection. Long-term facilitation induced by phosphorylated CREB1a occurred in the absence of short-term facilitation and was detectable only after an initial lag period of 4–6 hr. After that lag period, the facilitation gradually increased during the following 18–20 hr. The long-term increase in synaptic strength produced by the phosphorylated CREB1a was significantly larger than that induced by the unphosphorylated protein and required new protein and RNA synthesis. The finding that the injection of phosphorylated CREB1a did not induce short-term facilitation but was able to induce long-term facilitation indicates that CREB1a is the first, or at least an early, component in the cascade of gene expression responsible for long-term facilitation. Thus, the molecular events that initiate long-term facilitation most likely begin with the phosphorylation of CREB1a on S85 by PKA or by some other kinase. Consistent with this idea, injection of the mutated CREB1a S85A not only failed to induce, but completely blocked the long-term facilitation (Figure 5A).

Both CREB1a and CREB1c Are Phosphorylated In Vivo after Exposure to 5-HT

In *Aplysia* neurons in vivo, both CREB1a and CREB1c are phosphorylated in the basal state, and exposure of

intact *Aplysia* to 5-HT induces further phosphorylation (Figure 6). CREB1a phosphorylation in response to 5-HT exposure has two phases. First there is a transient phosphorylation that begins 10 min after exposure to 5-HT, peaks at 20 min, and returns to baseline by 40 min. This transient phosphorylation is not accompanied by an increase in the concentration of the CREB1a protein (Figure 6A). A second phase of phosphorylation emerges after 1 hr and increases for the next 12 hr, even after the 5-HT exposure was terminated. During this second phase, the increase in CREB1a phosphorylation is accompanied by an increase in the concentration of CREB1a protein. This increase in CREB1a concentration persists for at least 12 hr after terminating the exposure to 5-HT (Figure 6B) and is associated with an increase in the steady-state level of *CREB1* mRNA (Figure 6E), indicating that the second phase of CREB1a phosphorylation is likely to involve transcriptional and posttranscriptional modifications of the expression of CREB1a. In contrast, the concentration of CREB1a in neurons did not significantly change during a 12 hr incubation in the presence of either the RNA synthesis inhibitor actinomycin D or the protein synthesis inhibitor anisomycin (Figure 6F).

CREB1c protein is phosphorylated 40 min after 5-HT exposure in vivo, and this phosphorylation persists 8 hr after 5-HT withdrawal (Figures 6C and 6D). The persistent increase in CREB1c phosphorylation is not accompanied by the increase in CREB1c protein concentration. Thus, CREB1a and CREB1c protein expression and phosphorylation are differentially regulated in *Aplysia* neurons by transcriptional and posttranscriptional mechanisms.

CREB1b Is a Repressor of CREB1a and of Long-Term Facilitation

Although the long-term facilitation induced by injecting phosphorylated recombinant CREB1a was significant, its amplitude was 50% of that produced by five pulses of 5-HT. This suggests that additional molecular events may be involved in the transcriptional switch from short- to long-term facilitation. Since CREB1b and CREB1c, which are encoded by the *CREB1*β mRNA, might also be components of this switch, we tried to examine the roles of CREB1b and CREB1c proteins in sensory neurons during both short- and long-term facilitation.

As indicated above, CREB1b is a nuclear protein that has a leucine zipper and DNA-binding domain but lacks the activation domain of CREB1a. To examine the role of CREB1b in sensory neurons, we first injected recombinant CREB1b protein into sensory neurons and found that it significantly reduced long-term facilitation induced by five pulses of 5-HT as compared to control neurons exposed to five pulses of 5-HT and injected with buffer solution (Figures 7B and 7C). CREB1b injection did not affect short-term facilitation or basal synaptic transmission (Figure 7A).

We next injected an antisense oligonucleotide (As III/V) that specifically targets the boundaries between exons III and V in *CREB1*β mRNA and therefore interferes only with the expression of *CREB1*β mRNA. In contrast to the injection of As IV/V, which specifically targets the

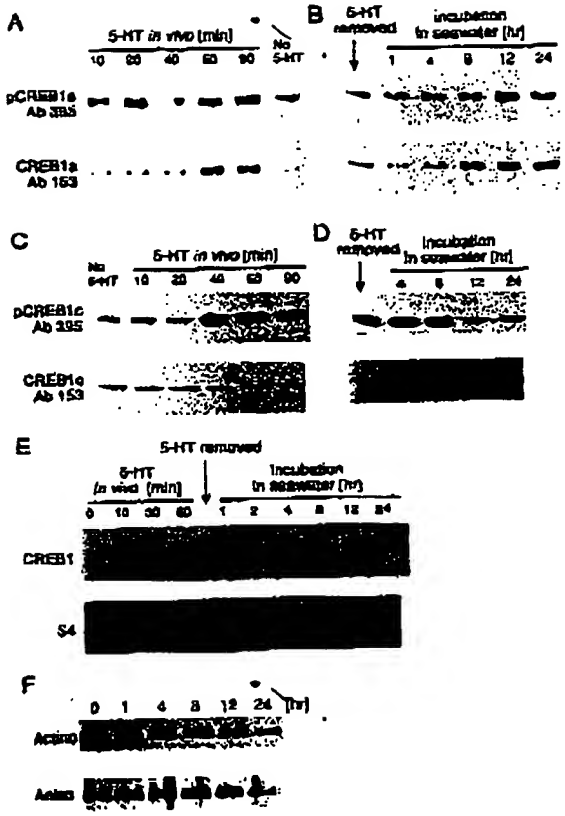

Figure 6. CREB1a and CREB1c Proteins Are Phosphorylated following Exposure to 5-HT In Vivo

Aplysia were exposed to 50 μM 5-HT in vivo for the times indicated (A and C) or first exposed to 50 μM 5-HT in vivo for 1 hr and then incubated in fresh seawater for the times indicated (B and D). Proteins isolated from *Aplysia* CNS (20 μg) were separated by SDS-PAGE, electroblotted, and probed with affinity-purified phospho-P-box CREB1 (395) antibodies and anti-P box CREB1 (153) antibodies.

(A and B) Brief exposure to 5-HT in vivo induces CREB1a phosphorylation in *Aplysia* neurons; 60 min long exposure to 5-HT increases both CREB1a phosphorylation and CREB1a protein concentration. CREB1a phosphorylation and expression increases and persists after 5-HT removal.

(C and D) Exposure to 5-HT in vivo induces CREB1c phosphorylation in *Aplysia* neurons without changing CREB1c protein concentration. CREB1c phosphorylation and expression persists 8 hr after 5-HT removal.

(E) Induction of CREB1a protein expression is accompanied by an increase in *CREB1* mRNA concentration. RNA was isolated from neurons of *Aplysia* exposed to 5-HT in vivo as in (A) and (B). *CREB1* mRNA expression was first determined in Northern blots with *CREB1a* cDNA and then reprobed with S4 cDNA to control for loading (Bartsch et al., 1995). Exposure to 5-HT increased the concentration of *CREB1* mRNA after 60 min of 5-HT exposure. The *CREB1* mRNA concentration further increased 1 hr after 5-HT exposure and persisted for 12 hr.

(F) CREB1a protein is stable for 12 hr in sensory clusters exposed to anisomycin or actinomycin D. *Aplysia* sensory clusters in artificial seawater were incubated with 50 μg/ml of actinomycin D (Actino) or 10 μM anisomycin (Aniso) for the time indicated. Protein extracts were isolated as in (A) and probed with anti-P box CREB1 (153) antibodies.

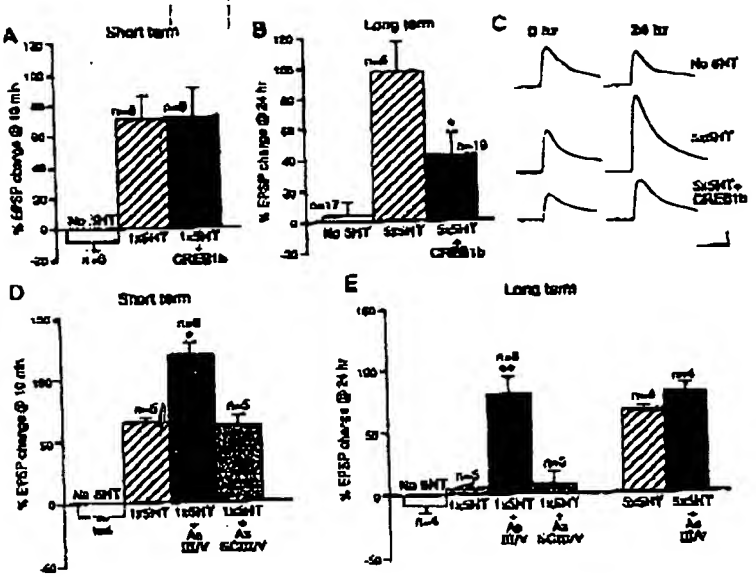

Figure 7. CREB1b Protein Is a Repressor of Long-Term Facilitation

(A and B) CREB1b injection does not affect short-term facilitation (A) but blocks long-term facilitation (B). The height of each bar corresponds to the mean percentage change ± SEM in EPSP amplitude tested 10 min after one pulse of 5-HT (A) or 24 hr after five pulses of 5-HT (B). *, $p < 0.05$ compared to noninjected cells exposed to five pulses of 5-HT.

(C) Examples of EPSPs recorded at indicated time points in neurons injected with CREB1b. Calibration bars are 10 mV; 50 ms.

(D) Injection of As III/V antisense oligonucleotides targeting *CREB1β* mRNA increases short-term facilitation induced by a single pulse of 5-HT. The height of each bar corresponds to the mean percentage change ± SEM in EPSP amplitude tested 10 min after one pulse of 5-HT. *, $p < 0.05$ compared to both noninjected sensory neurons and neurons injected with scrambled oligonucleotide (As SCIII/V).

(E) Injection of anti-*CREB1β* antisense oligonucleotide AS III/V into sensory neurons paired with a single pulse of 5-HT induces long-term facilitation. The height of each bar corresponds to the mean percentage change ± SEM in EPSP amplitude tested 24 hr after a single pulse of 5-HT. *, $p < 0.01$ compared to noninjected cells and cells injected with the scrambled As SCIII/V oligonucleotide.

CREB1α mRNA, the injection of As III/V did not inhibit long-term facilitation when paired with five pulses of 5-HT. In fact, the injection of As III/V lowered the threshold for long-term facilitation (Figure 7E). Thus, whereas a single pulse of 5-HT produced only a transient short-term facilitation in uninjected cells or in cells injected with scrambled oligonucleotide (As SCIII/V), a single pulse of 5-HT produced full long-term facilitation in cells injected with As III/V (Figure 7E). These data indicate that bicistronic *CREB1β* mRNA encodes a CREB1b protein that is a repressor of CREB1a-mediated transcription and of long-term facilitation.

CREB1c Is a Cytoplasmic Modulator for Both Short-Term and Long-Term Facilitation

In the course of studying the function of CREB1b, we made the surprising finding that the expression of *CREB1β* mRNA not only modulates long-term facilitation but also can modify short-term facilitation.

We first found this modulation in the course of injecting the antisense oligonucleotide (As III/V) that targets the *CREB1β* mRNA. When this injection was followed by one pulse of 5-HT, it almost doubled the amplitude of the short-term facilitation as compared to the effect of a single pulse of 5-HT in control noninjected neurons or in neurons injected with scrambled As SCIII/V oligonucleotide (Figure 7D). How could this antisense oligonucleotide, directed against *CREB1β* mRNA, affect the short-term process? Blocking the translation of one reading frame in bicistronic mRNAs often increases the translation of the other one (Kozak, 1986a). We therefore wondered whether, rather than simply reducing the translation of the CREB1b protein, the As III/V oligonucleotide could also enhance the translation of CREB1c from the bicistronic *CREB1β* mRNA. Since our antibodies do not allow us to measure the effects on CREB1c expression of antisense injections by immunochemistry, we attempted to address this issue by injecting the purified recombinant CREB1c protein into the sensory neurons and monitoring both short- and long-term facilitation.

To determine whether CREB1c enhances short-term facilitation, we injected recombinant CREB1c peptide, either phosphorylated by PKA on S85 or unphosphorylated, into sensory neurons. The injection of phosphorylated or unphosphorylated CREB1c had no effect on basal synaptic transmission or on short- or long-term facilitation (data not shown). By contrast, injection of unphosphorylated CREB1c followed by a single pulse of 5-HT doubled the normal amplitude of short-term facilitation evident at 10 min as compared to control, buffer-injected neurons (Figure 8A). In further contrast to control cells, the injection of unphosphorylated CREB1c paired with one pulse of 5-HT also induced long-term facilitation at 24 hr (Figure 8B). Thus, in response to a single pulse of 5-HT, the unphosphorylated CREB1c can facilitate both the short- and the long-term process. By contrast, the injection of the phosphorylated CREB1c followed by a single pulse of 5-HT had no effect on either short-term or long-term facilitation (Figures 8A and 8B).

Although CREB1a is colinear with CREB1c up to R87, injection of the CREB1a protein into the cytoplasm of sensory neurons had no effect on short-term facilitation. This difference in activity between CREB1c and CREB1a may be due to the absence of the C-terminal DNA binding and dimerization domain in CREB1c, to differences in the subcellular localization of the two proteins, or to

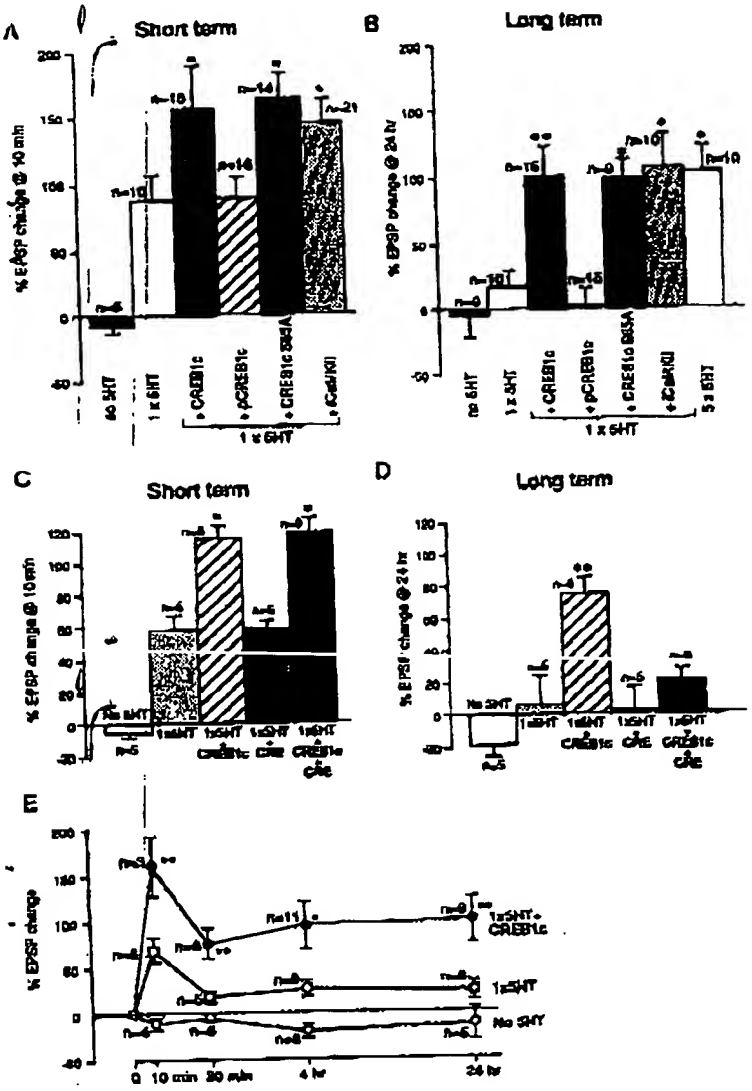

Figure 8. CREB1c Protein Modulates Both Short- and Long-Term Facilitation, and the Induction of Long-Term Facilitation by CREB1c Protein Paired with One Pulse of 5-HT Requires the CRE Binding Activity of CREB1a

(A and B) Injection of either unphosphorylated CREB1c or CaMKII autoinhibitory peptide inhibitor enhances short-term facilitation and induces long-term facilitation when paired with one pulse of 5-HT. The height of each bar corresponds to the mean percentage change ± SEM in EPSP amplitude following injection of recombinant unphosphorylated CREB1c (CREB1c), mutant CREB1c S85A (CREB1c S85A), and phosphorylated CREB1c (pCREB1c) or CaMKII autoinhibitory peptide (iCaMKII) tested 10 min (A) or 24 hr (B) after a single pulse of 5-HT. *, $p < 0.05$ compared to noninjected or phosphorylated CREB1c injected neurons exposed to one pulse of 5-HT (A); *, $p < 0.05$; **, $p < 0.01$ compared to the cells treated with one pulse of 5-HT (B).

(C) Injection of CRE oligonucleotide into sensory neurons coinjected with CREB1c protein did not affect the short-term facilitation increase induced by CREB1c only. Bar graphs represent the effect of CREB1c and CRE injection on short-term facilitation. The height of each bar corresponds to the mean percentage change ± SEM in EPSP amplitude tested 10 min after one pulse of 5-HT treatment. *, $p < 0.05$ compared to noninjected cells or cells injected with CRE only and exposed to one pulse of 5-HT.

(D) CRE injection blocks the long-term facilitation caused by CREB1c injection paired with one pulse of 5-HT. Bar graph represents the effect of CRE and CREB1c injection on long-term facilitation. The height of each bar corresponds to the mean percentage change ± SEM in EPSP amplitude tested 24 hr after one pulse of 5-HT treatment. **, $p < 0.01$ compared to noninjected cells, cells injected with CRE, or cells coinjected with CREB1c and CRE.

(E) Time course of facilitation following injection into sensory neurons of unphosphorylated CREB1c protein followed by one pulse of 5-HT. Each time point represents the EPSP amplitude changes ± SEM. For each time point, the EPSP amplitude increase was significantly higher in cells injected with CREB1c and treated with one pulse of 5-HT compared to uninjected cells exposed one pulse of 5-HT. *, $p < 0.05$; **, $p < 0.01$.

effects of the twelve C-terminal amino acids present only in CREB1c.

CREB1a and CREB1c Act Coordinately to Regulate the Transition from Short- to Long-Term Facilitation

The injection of phosphorylated CREB1a without 5-HT exposure induces long-term facilitation without inducing the short-term. Since the injection of CREB1c paired with a single pulse of 5-HT is sufficient to enhance short-term facilitation and induce long-term facilitation, we asked whether the long-term facilitation induced by CREB1c requires DNA binding activity of CREB1a. We therefore coinjected the CREB1c protein and the CRE oligonucleotide and paired the injection with one pulse of 5-HT. This coinjection led to a significant enhancement of short-term facilitation but did not induce long-term facilitation (Figures 8C and 8D). Thus, the induction of long-term, but not the enhancement of short-term facilitation by CREB1c paired with a single pulse of 5-HT requires CREB1a DNA binding activity. This experiment supports the idea that CREB1c modulates the action of CREB1a but is not sufficient to induce long-term synaptic changes. As a corollary, these findings indicate that CREB1c and CREB1a act coordinately to initiate the long-term process.

The Induction of Both Short- and Long-Term Facilitation by Dephosphorylated CREB1c Appears to Parallel Inhibition of Calcium/Calmodulin-Activated CaMKII

Although PKA and PKC phosphorylate both CREB1c and CREB1a on S85, CREB1c, unlike CREB1a, is not phosphorylated on S85 by CaMKII in vitro. We found that both the dephosphorylated wild-type and the mutated S85A CREB1c inhibit CaMKII activity in vitro (38.4% ± 3.6% and 42.7% ± 5.0% inhibition by CREB1c and CREB1c S85A as compared to 52.3% ± 6.8% inhibition by CaMKII autoinhibitory peptide, both at 40 μM, and 19.0% ± 3.1% and 23.5% ± 4.8% by CREB1c and CREB1c S85A as compared to 26.6% ± 4.0% inhibition by CaMKII autoinhibitory peptide, both at 8 μM). The recombinant CREB1c phosphorylated by PKA in vitro inhibits purified CaMKII significantly less (4.1% ± 2.2% at 40 μM; 2.8% ± 3.2% at 8 μM). Similarly, unphosphorylated recombinant wild-type CREB1c and mutated CREB1c S85A inhibit CaMKII activity in *Aplysia* neuronal extracts (68.3% ± 2.4% inhibition by CREB1c and 69.5% ± 4.2% inhibition by CREB1c S85A, both 40 μM). The PKA-phosphorylated CREB1c inhibits CaMKII activity in *Aplysia* neuronal extracts significantly less (22.1% ± 6.8% inhibition at 40 μM). Neither unphosphorylated nor PKA-phosphorylated CREB1c affected PKA or PKC activity in vitro or in *Aplysia* neuronal extracts.

To ask whether the CREB1c could interfere with calmodulin activation of CaMKII in *Aplysia* neurons, we compared the physiological effect of injecting CREB1c to that of injecting the autoinhibitory peptide of CaMKII. As with the injection of unphosphorylated CREB1c and CREB1c S85A mutant, but not the PKA-phosphorylated CREB1c, injection of the autoinhibitory peptide of CaMKII into the *Aplysia* sensory neurons followed by one pulse of 5-HT increased short-term facilitation and induced long-term facilitation (Figures 8A and 8B) as compared to uninjected cells. Thus, the unphosphorylated CREB1c parallels the calmodulin-binding CaMKII inhibitory peptide in blocking CaMKII activity in vitro and lowering threshold for both long- and short-term facilitation in sensory neurons. Phosphorylation of CREB1c by PKA abolishes both of these effects.

Discussion

There is increasing evidence that the transcriptional activator CREB is important for long-term synaptic plasticity and long-term memory formation in *Aplysia*, *Drosophila*, and mice (Dash et al., 1990; Bourtchuladze et al., 1994; Yin et al., 1994, 1995a). However, it has not previously been possible to demonstrate which specific CREB/ATF1- or CREM isoforms participate in any of these instances of synaptic plasticity and memory storage. We have found that this problem may be simplified in *Aplysia* because there appears to be only one member of the CREB, CREM, and ATF1 family of genes: CREB1. We have characterized the specific isoforms of CREB1 that are expressed in *Aplysia* sensory neurons and manipulated the expression of these isoforms individually. We find that the *CREB1* gene encodes in the sensory neurons two alternatively spliced mRNAs, *CREB1α* and *CREB1β*, that are translated into three different proteins, CREB1a, CREB1b, and CREB1c, that mediate a coordinated and temporally sequenced program. In this program, CREB1a serves as an activator of the long-term process, CREB1b as a repressor of the long-term process, and CREB1c as a cytoplasmic regulator of both the short-term and the CREB1a-mediated long-term processes.

CREB1a Activation Is Necessary and Sufficient for Long-Term Facilitation

Recent studies in rodent hippocampal neurons indicate that CREB phosphorylation can be induced by a wide range of frequencies of electrical stimulation. Both low frequencies inducing LTD and high frequencies inducing LTP and intermediate frequencies that have no physiological effect at all induce comparable CREB phosphorylation (Bito et al., 1996; Deisseroth et al., 1996). Our data provide evidence that the phosphorylation of CREB1a is both necessary and sufficient for the induction of long-term facilitation in *Aplysia* neurons and is likely a first step in the transcriptional switch from the short-term to the long-term process. CREB1a phosphorylation increases in *Aplysia* neurons following in vivo exposure to 5-HT, and interfering with CREB1a expression by injecting antibodies or antisense oligonucleotides selectively blocks long-term facilitation. Injection of recombinant phosphorylated CREB1a into sensory neurons produces about 50% of the induced long-term facilitation by five pulses of 5-HT in the absence of 5-HT. Following injection of CREB1a, a single pulse of 5-HT, which normally induces short-term facilitation, induced long-term facilitation comparable to that induced by five pulses of 5-HT. This experiment is the cellular counterpart of the behavioral experiments by Yin and Tully in *Drosophila* that showed that overexpression of the CREB2a activator can lead to long-term memory formation when paired with a single training trial (Yin et al., 1995a).

CREB1a Expression Increases following Extended 5-HT Exposure

Previous studies focused on the role of CREB1 proteins in the initial switch to turn on long-term neuronal plasticity. Our data suggest that CREB1a is not only a key element in initiating the switch but may also have a role in the maintenance of long-term facilitation.

We found that upon exposure to 5-HT in vivo CREB1a is phosphorylated in at least two phases. The first phase, which occurs within 10–15 min after exposure to 5-HT, peaks at 20 min and returns to baseline within 40 min. This increase in CREB1a phosphorylation at S85 is not accompanied by an increase in the concentration of the CREB1a protein and therefore most likely reflects the sequential activation of 5-HT receptor, adenylyl cyclase, and PKA.

However, if animals are exposed to 5-HT continuously for 1 hr—a procedure that gives rise to long-term sensitization in the animal—a second phase of CREB1a phosphorylation is induced, which now persists for at least 24 hr. This phosphorylation is detectable at 1 hr and is accompanied by an increase in the concentration of CREB1a protein. This suggests that CREB1a expression

may be autoregulated, perhaps directly by CREB1a activating the CRE regulatory sequences in the *CREB1* gene. Earlier studies have indicated that mammalian *CREB* mRNA expression is induced following prolonged exposure to the antidepressant rolipram, a cAMP phosphodiesterase inhibitor, or following exposure to drugs of abuse (Nibuya et al., 1996; Widnell et al., 1996).

CREB1b Is a Repressor Similar to Mammalian ICER and I-CREB

The CREB1b polypeptide translated from the alternatively spliced *CREB1β* mRNA contains the bZIP domain but lacks the N-terminal activation domain and the P box of CREB1a. This CREB1b protein resembles the mammalian ICER and I-CREB repressors structurally and functionally (Molina et al., 1993; Walker et al., 1996). Consistent with its structure, *Aplysia* CREB1b forms homodimers or heterodimers with CREB1a. These dimers bind to CREs and inhibit CREB1a-mediated transactivation in F9 cells. Previously, we cloned the *CREB2* gene (Bartsch et al., 1995), which is structurally unrelated to CREB1. CREB2 also represses CREB1-mediated transactivation in F9 cells and is a repressor of long-term facilitation. Thus, CREB1b and CREB2 may represent parallel inhibitory pathways for regulating CREB1a-mediated gene activation in *Aplysia* sensory neurons.

CREB1c Is a Cytoplasmic Regulator

One of the most interesting components of the *Aplysia* CREB1 regulatory unit, and the least well understood, is the cytoplasmic regulator CREB1c. CREB1c is a cytoplasmic protein with no direct transcriptional activity. It lacks the DNA-binding domain, the dimerization domain, and the nuclear localization signal of CREB1a.

Injection of recombinant CREB1c significantly enhances both long-term and short-term facilitation. CREB1c does not induce long-term facilitation by itself, but requires the pairing with a 5-HT pulse. In addition, the DNA binding activity of CREB1a is necessary for CREB1c-induced long-term facilitation. This long-term facilitation can be blocked by the CRE oligonucleotide, with which CREB1c does not interact directly, thus indicating that CREB1c induces long-term facilitation through CREB1a.

Our data also suggest differential interaction of CREB1a and CREB1c with calmodulin and CaMKII. The splicing that generates CREB1c modifies its P box as compared to CREB1a. It removes the S94, homologous to S142 in mammalian CREB1. Phosphorylation in mammals at S142 by CaMKII is inhibitory to CREB transactivation mediated by PKA or CaMKIV (Sun et al., 1994). CREB1c is phosphorylated by PKA and PKC, but although the consensus phosphorylation site for CaMKII at S85 in CREB1c remains intact, it is not readily phosphorylated by CaMKII in vitro. In fact, CREB1c inhibits CaMKII activity in vitro. Although the injection of CREB1c increases both long-term and short-term facilitation in parallel to the injection of CaMKII autoinhibitory peptide, further evidence is necessary to determine whether the facilitatory effect of CREB1c is mediated through its interaction with the calmodulin/CaMKII pathway in vivo.

How is the CREB1c regulated? Upon exposure to 5-HT, CREB1c is phosphorylated in vivo but with slower kinetics than CREB1a. Since this phosphorylation turns off the facilitatory actions of CREB1c, the phosphorylation of CREB1c may serve as a termination signal that serves to increase the threshold for subsequent signals once an action of 5-HT has been initiated. Thus, CREB1c appears to be a modulator of PKA- and CREB1a-mediated transcription, not by acting on the transcriptional process itself, but rather by modifying the core cytoplasmic signal transduction pathways activating CREB1a. Although the cytoplasmic location of a CREB1c isoform is surprising, it is by no means unique to *Aplysia*. Mammalian CREB W and some forms of CREBα are similarly cytoplasmic (Wasner et al., 1991; Hermanson et al., 1996). The role of the cytoplasmic CREB1 isoforms in mammals and *Drosophila* remains to be elucidated, but our data and the conservation of this splicing pattern among species suggest that they are likely to play a role in CREB1-mediated gene regulation as well as in synaptic plasticity.

Experimental Procedures

General Methods

Standard manipulations of *E. coli*, *S. cerevisiae*, proteins, and nucleic acids were performed essentially as described (Harlow and Lane, 1988; Ausubel et al., 1993; Bartsch et al., 1995).

Plasmids, Cloning, and Transient Transfections and Reporter Assays

Subcloning of *CREB1* cDNAs was done by PCR using Pfu polymerase (Stratagene). The initiation codons of the three ApCREB-1 isoforms were replaced by NcoI restriction sites. The CREB1 isoforms were cloned in the modified pET-30 for expression in *E. coli* and in pRcRSV (Invitrogen). pRcRSV-PKA C-α1 expressing the PKA catalytic subunit was generously provided by R. Goodman. The luciferase reporter pGL3-6xCRE and transient transfection assays in F9 cells were described previously (Bartsch et al., 1995).

Immunocytochemistry

CREB1c and CREB1b ORF with N-terminal 6His tags were subcloned from pET30 into pRcRSV and transfected into F9 cells with pRSV-lacZ reporter plasmid. The F9 cells were immunostained with anti-6-His antibody (BABCO) 72 hr after transfection as described for *Aplysia* neurons (Martin et al., 1997). The cells transfected with only the pRSV-lacZ plasmid did not show any staining.

Aplysia CNS cDNA and Genomic Libraries Construction and Screening

The *Aplysia* CNS cDNA and genomic libraries were constructed in λ ZAP and λ FIX (Stratagene), respectively. Two partial CREB1 cDNA clones were initially isolated by hybridization with rat *CREB* cDNA (generously provided by R. Goodman) at Tm-35°C. The full-length *CREB1* cDNAs and genomic clones were isolated by subsequent high stringency hybridization screening of corresponding libraries. A total of 13 cDNA clones (10 corresponding to *CREB1β* mRNA and 3 to *CREB1α* mRNA) and 15 genomic clones were sequenced.

Oligonucleotides

DNA oligonucleotides were synthesized (GIBCO-BRL) and purified on OPC columns. The sequences of the injected antisense oligonucleotides are: As I/II (GCCTTCTGACATGTCAATTAC), As III (CAAAATTTTCCTGTACGAAG), As IV (GGATACTGGAGAGGACA CTC), and AS III/V (GTTTGGACATCTCTACGAAG). The scrambled control oligonucleotides (SC) are reversed, not complementary, sequences. The oligonucleotides used in RT-PCR are: E5 (TTTGACC GAAGGCCTTCGT), E6 (TAACAATGACAAACGCTAGTCCAA), and E8 (CTGATAGGCTTGTACATTTGT).

Electrophoretic DNA Mobility Shift Assays

The gel-shift assays with the CRE oligonucleotide were performed as described previously (Dash et al., 1990; Bartsch et al., 1995).

Purification of Recombinant Proteins

6×His-CREB1 fusion proteins were expressed and purified using the QIAexpress system (Qiagen, denaturing protocol). The bound 6×His-CREB1 proteins were renatured stepwise on the Ni-NTA resin, eluted with 250 mM imidazole, and dialyzed.

Antisera Production and Affinity Purification

Two polyclonal rabbit antisera were raised (BABCO) against recombinant 6×His-CREB1a. Both antibodies produced similar results in microinjection experiments, and only the results with the R1 antibody are presented. The R1 antibody recognizes all three recombinant CREB1a, CREB1b, and CREB1c proteins. Polyclonal antisera were also raised against synthetic peptides KRREILTRRPSYR (antiserum 153) and KRREILTRRPS(PO$_3$)YR (antiserum 395) conjugated to KLH. The R1 antibodies and 153 antibodies were affinity purified on resins made by coupling the 6×His-CREB1a to mixed Affi-Gels 10 and 15 (BioRad). The anti-phospho-CREB1 antibody 395 was affinity purified on the phosphopeptide 395 affinity resins using the EDC/DAP^3 kit (Pierce) and was blocked by the unphosphorylated bacterially expressed CREB1a protein. The blocked 395 antibody recognizes only phosphorylated CREB1.

Aplysia CNS Protein and RNA Preparation

Aplysia were exposed in vivo to 50 μM 5-HT and then sacrificed or returned to seawater. Following 5-HT exposure, the dissected ganglia were immediately lysed in Trizol (GIBCO) containing 4 M guanidine/HCl. This procedure minimizes both protein degradation and preserves CREB1 phosphorylation. RNA and protein were isolated in parallel from the nervous system. The RNA was isolated from the water phase according to the manufacturer and analyzed by Northern blots. To minimize protein degradation and to preserve CREB1 phosphorylation, the acetone-precipitated protein from the phenol phase was redissolved in 6 M urea, 65 mM Tris (pH 6.8), 4% SDS, and analyzed by Western blots as described (Bartsch et al., 1995).

Subcellular Fractionation

The central nervous systems from two *Aplysia* were carefully desheathed and the ganglia were incubated in 0.6% NP-40 in artificial seawater for 15 min on ice with occasional gentle agitation. After centrifugation at 1250 × g for 5 min at 4°C, the supernatant was transferred to a new tube and the nuclear pellet was examined under a microscope. From both fractions, RNA, DNA, and protein were isolated using Trizol (GIBCO) using manufacturer protocol. Ethidium bromide staining in an agarose gel verified that DNA was present only in the nuclear fraction.

RNA Extraction from Sensory Neuron Cultures and RT-PCR

RT-PCR, protein extraction, and Western blotting were done as described previously (Bartsch et al., 1995). To maintain a linear range of amplification with primers E3 and E6, we have used 15 cycles of amplification combined with hybridization with the E5 oligonucleotide.

Kinase Assays and Phosphorylation of CREB1 Isoforms

Aplysia CNS was homogenized in 20 mM Tris (pH 7.5), 10 mM mercaptoethanol, 25 mM NaF, 1 mM EDTA, 0.25 mM EGTA, 20 μM P176, 20 μM P90, 23 μg/ml AEBSF, 1 μg/ml aprotinin, 0.5 μg/ml leupeptin, and 5 mM benzamidine and then centrifuged at 20,000 × g for 15 min at 4°C. Diluted extract was incubated with 2 μg syntide II and various concentrations of CREB1c or CaMKII peptide inhibitor (Ala^{286}) in a buffer containing 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, 100 μM ATP including 1 μCi [^{32}P]ATP, 2 mM $CaCl_2$, and 1 μM calmodulin for 5 min at 25°C. The reaction mixture was spotted on P81 paper (Whatman), washed in 1% phosphoric acid, dried, and scintillation counted. The effect of CREB1c on purified mouse CaMKII (kindly provided by M. Mayford) was assayed identically.

Recombinant CREB1 isoforms were phosphorylated by PKA (Sigma) while immobilized on the Ni-NTA resin and then washed extensively with 6 M guanidium/HCl, renatured gradually in TBS, and then eluted with 150 mM imidazole and dialyzed.

Aplysia Cell Culture and Electrophysiology

Aplysia cell cultures and electrophysiology were done as described previously (Alberini et al., 1994; Bartsch et al., 1995).

Induction of Facilitation, Antibody, DNA Oligonucleotides, and Peptide Injection

Two protocols were used to induce synaptic facilitation in the *Aplysia* cultures. In short-term training, after testing the initial EPSP amplitude, 10 μM 5-HT was applied for 5 min (single pulse). The EPSP was retested after 10 min (short-term facilitation) and at various later time points up to 24 hr (long-term facilitation) after the washout of the 5-HT. In long-term training, the cultures were exposed to five pulses of 10 μM 5-HT for 5 min each at 20 min intervals (five pulses). The amount of facilitation was calculated as the percentage change in EPSP amplitude recorded before and at the indicated time points after exposure to 5-HT. When the posttreatment EPSP evoked an action potential, a value of 80 mV was used for quantitation. The antibodies in injection buffer (1 mg/ml; Alberini et al., 1994) were pressure injected into the sensory neurons 1 hr before 5-HT treatment. The antisense oligonucleotides diluted in the same buffer (50 μg/ml) were injected 4 hr before 5-HT exposure unless indicated otherwise. The [Ala^{286}] CaMKII inhibitor peptide (Calbiochem #208710, 40 μM) was injected 1 hr before the 5-HT exposure. Where indicated, anisomycin (10 μM) or actinomycin D (50 μg/ml) was added to the culture medium 1 hr before the 5-HT exposure as described (Montarolo et al., 1986). All data are presented as mean percentage change ± SEM in EPSP amplitude after 5-HT treatment, compared with the initial pretreatment amplitude. One-way analysis of variance and Newman-Keuls multiple range test were used to determine the significance of the EPSP changes. In all experiments, the basal synaptic transmission was not affected by injection of antibodies, oligonucleotides, or CREB1 proteins.

Acknowledgments

We thank Richard Axel, Amy Chen, Thomas Jessell, Kelsey Martin, and Chris Pittenger for critical discussion and helpful comments during the preparation of the manuscript; Harriet Ayers and Irma Trumbor for typing the manuscript; and Chuck Lam for the graphic design of the figures. This research was supported by the Howard Hughes Medical Institute and by the National Institutes of Health grants MH45923-09.

Received April 28, 1998; revised September 3, 1998.

References

Alberini, C.M., Ghirardi, M., Metz, R., and Kandel, E.R. (1994). C/EBP is an immediate-early gene required for the consolidation of long-term facilitation in Aplysia. Cell 76, 1099–1114.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1993). Current Protocols in Molecular Biology (New York: John Wiley and Sons, Inc.).

Bailey, C.H., and Chen, M. (1983). Morphological basis of long-term habituation and sensitization in Aplysia. Science 220, 91–93.

Bailey, C.H., Bartsch, D., and Kandel, E.R. (1996). Toward a molecular definition of long-term memory storage. Proc. Natl. Acad. Sci. USA 93, 13445–13452.

Bartsch, D., Ghirardi, M., Skehel, P.A., Karl, K.A., Herder, S.P., Chen, M., Bailey, C.H., and Kandel, E.R. (1995). Aplysia CREB2 represses long-term facilitation: relief of repression converts transient facilitation into long-term functional and structural change. Cell 83, 979–992.

Bito, H., Deisseroth, K., and Tsien, R.W. (1996). CREB phosphorylation and dephosphorylation: a Ca^{2+}- and stimulus duration–dependent switch for hippocampal gene expression. Cell 87, 1203–1214.

Bliss, T.V., and Collingridge, G.L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361, 31–39.